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Report

Natural Variation in Leaf Morphology Results from Mutation of a Novel *KNOX* Gene

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Summary

Striking diversity in size, arrangement, and complexity of leaves can sometimes be seen in closely related species. One such variation is found between wild tomato species collected by Charles Darwin from the Galapagos Islands [1–5]. Here, we show that a single-nucleotide deletion in the promoter of the *PETROSELINUM* (*PTS*) [3] gene upregulates the gene product in leaves and is responsible for the natural variation in leaf shape in the Galapagean tomatoes. *PTS* encodes a novel *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) gene that lacks a homeodomain. We also showed that the tomato classical mutant *bipinnata* (*bip*) [6], which recapitulates the *Pts* phenotype, results from the loss of function of a *BEL-LIKE HOMEODOMAIN* (*BELL*) gene, *BIP*. We used bimolecular fluorescence complementation and two-hybrid competition assays to show that PTS represses KNOX1 protein interactions with BIP, as well as subsequent nuclear localization of this transcriptional complex. We suggest that natural variation in leaf shape can be created with a rheostat-like mechanism that alters the KNOX1 protein interaction network specifically during leaf development. This subtle change in interaction between transcription factors leaves essential KNOX1 function in the shoot apical meristem intact and appears to be a facile way to alter leaf morphology during evolution.

Results and Discussion

Petroselinum Is Responsible for the Natural Variation in Leaf Shape in the Galapagean Tomatoes

The most conspicuous characteristic of leaf shape is the degree to which the leaf is subdivided into smaller segments. Leaves lacking subdivision are termed simple, whereas divided leaves are termed compound. The *Lycopersicon* section of the *Solanum* genus is composed of 13 species, including cultivated tomato, all of which produce compound leaves that vary greatly in the degree of leaf dissection [7, 8]. The most dramatic example of this variation was identified by J.G. Hooker between two accessions, *Solanum cheesmaniae* (previously known as *Lycopersicon cheesmanii* f. *major*) and *Solanum galapagense* (previously known as *Lycopersicon cheesmanii* f. *minor*), collected by Charles Darwin from the Galapagos Islands [1, 2]. The two species are fully sexually compatible, yet exist as distinct populations in the wild, rarely hybridizing and inhabiting distinct habitats [4]. Despite the relatively recent divergence of the two species, *S. galapagense* displays several morphological novelties relative to other wild tomato species. The most obvious of these is the unique leaf shape. The *S. cheesmaniae* leaf is unipinnately compound, resembling cultivated wild-type tomato (*S. lycopersicum*) and the presumed ancestor of both the Galapagean tomatoes (*S. pimpinellifolium*), whereas the leaves of *S. galapagense* have increased complexity, usually producing three orders of leaflets [4] (Figures 1A–1C). The increased-leaf-dissection phenotype is completely fixed in *S. galapagense* and has not been reported to occur in populations of *S. cheesmaniae* [2, 5]. Introgression of the trait from *S. galapagense* into cultivated wild-type tomato (VF36) demonstrated that this phenotype is conferred by a single semidominant locus, named *Petroselinum* (*Pts*) for its phenotypic resemblance to parsley [3] (Figure 1D). The *Pts* phenotype is characterized by increased primary- and secondary-leaflet production, in addition to development of tertiary and quaternary leaflets not observed in the wild-type (Figures 1C and 1D). *Pts* also shows increased marginal serration. Early stages of leaf development (P1–P5) in *Pts* and the wild-type are indistinguishable by scanning electron microscopy (SEM) (Figures 1E and 1F). However, at the later leaf-expansion stage, *Pts* leaves show increased primary-leaflet initiation and marginal serration compared to the wild-type (Figures 1G and 1H).

Map-Based Cloning of *Pts*

To identify the molecular basis for the *Pts* phenotype, we performed a large-scale linkage analysis (Figures 2A–2D). The *Pts* mutation was delineated to a 1749 bp interval between the markers mP3 and mP4 (Figure 2C). Extensive sequence comparison of this region between the wild-type and *Pts* revealed four SNPs and a 1 bp deletion (Figure 2D and Figure S1 available online). To determine which polymorphism is responsible for the phenotype, we compared the sequence of this region in nine accessions of both *S. cheesmaniae* and *S. galapagense*. All of the four single-nucleotide polymorphisms (SNPs) were identical between *S. cheesmaniae* and *S. galapagense*. However, the 1 bp deletion was specific to *Pts* and *S. galapagense*, indicating that this deletion could be responsible for the increased-leaf-complexity phenotype. Sequence homology searches revealed that the 1 bp deletion is 1266 bp upstream of an open reading frame (ORF) corresponding to a previously registered sequence, *TKD1* (*TOMATO KNOX-LIKE HOMEODOMAIN PROTEIN* 1) (AF375969). We isolated full-length complementary DNA (cDNA), and the ORF encodes a predicted product of 171 amino acid residues. Henceforth, we refer to this gene as *PTS/TKD1*.

Because the *Pts* mutation was found in the promoter region of the *PTS/TKD1* gene, we compared the expression level of this gene between wild-type and *Pts* (Figure 2E). In leaves, the expression of *PTS/TKD1* was higher in *Pts* than in the wild-type. Additionally, *PTS/TKD1* was overexpressed in leaves of *S. galapagense* compared with those of *S. cheesmaniae* (Figure 2F). Thus, the natural variation in leaf complexity between *S. galapagense* and *S. cheesmaniae* is likely conferred by overexpression of *PTS/TKD1* in developing leaves. The fact that *Pts* is a semidominant mutant suggests that it is a dosage-sensitive regulator of leaf complexity. In situ hybridization analysis showed that *PTS/TKD1* was strongly expressed in the shoot apical meristem and the adaxial domain

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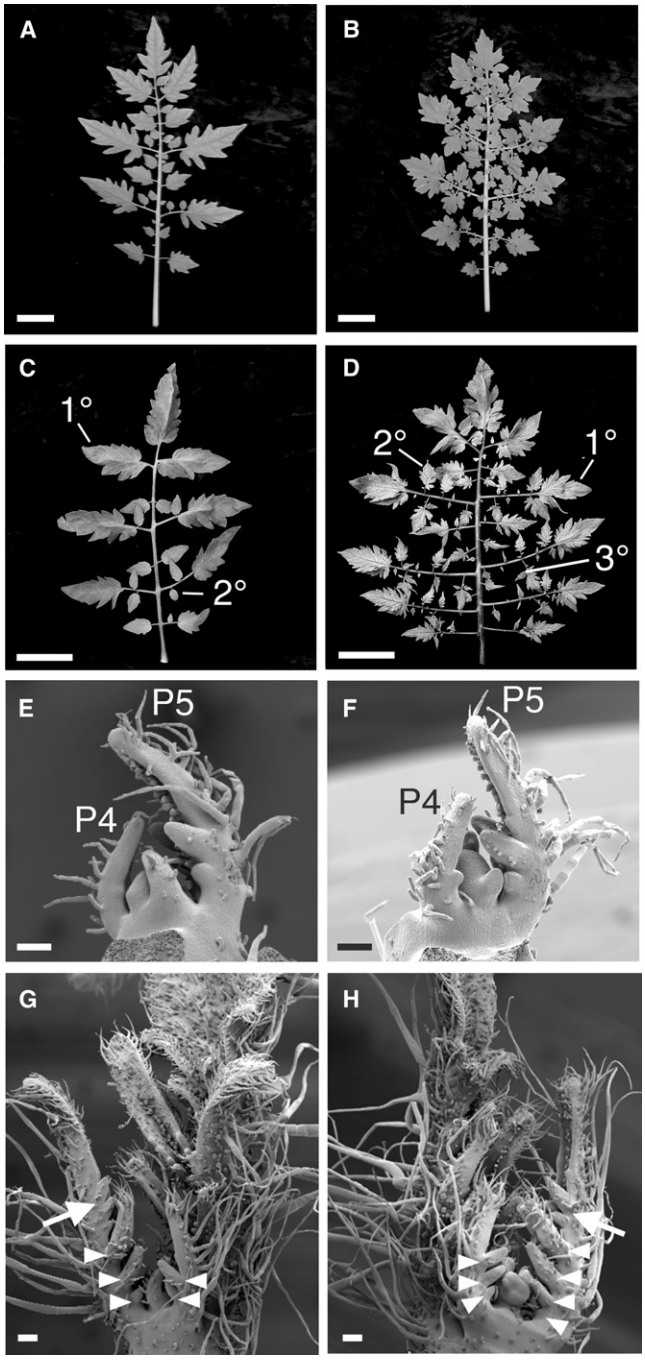

Figure 1. Leaf Phenotypes

(A) Leaf from *Solanum cheesmaniae*.
(B) Leaf from *Solanum galapagense*.
(C) Leaf from cultivated wild-type tomato (VF36).
(D) Leaf from *Pts*.
(E–H) SEM images of shoot apices of wild-type (E and G) and *Pts* (F and H). Fourteen-day-old (E and F)and 21-day-old (G and H) tomato plants are shown. Arrowheads and arrows in (G) and (H) indicate the position of leaflet primordia and lobes, respectively.
1°, 2°, and 3° represent primary, secondary, and tertiary leaflets, respectively. Scale bars represent 2.5 cm (A and B), 6 cm (C and D), 100 μm (E and F), and 200 μm (G and H).

of the developing leaf, particularly in the initiating leaflet primordia, suggesting the possibility that *PTS/TKD1* is involved in leaflet formation (Figure S2).

To further confirm the identity of the *Pts* mutation, we transformed wild-type tomato with the 3 kb promoter sequence from *Pts* fused to the coding sequence from the wild-type (*pPts::PTS/TKD1*). These transgenic tomato plants showed a clear *Pts* phenotype (Figure 2G). Transgenic tomato transformed with the wild-type promoter and coding sequence (*pPTS::PTS/TKD1*) also showed moderate *Pts* phenotype, and the phenotype was correlated with an increase in the expression level of *PTS/TKD* (Figure S3). These results confirm that overexpression of the *PTS/TKD1* gene product causes the *Pts* phenotype, and the severity of *Pts* phenotype depends on the dosage of PTS/TKD1.

PTS Encodes a Novel *KNOX* Gene that Lacks a Homeodomain

PTS/TKD1 has homology to the class 1 *KNOTTED-LIKE HOMEOBOX* (*KNOX1*) genes that encode homeodomain-containing transcription factors [9]. The *KNOX1* genes are expressed in the plant shoot apical meristem (SAM), where they function to maintain indeterminacy of the vegetative stem cell niche [9]. In the majority of compound-leafed species, *KNOX1* genes are also expressed in the developing leaf primordia, where they function to promote leaflet initiation [10, 11]. Overexpression of *KNOX1* genes in compound-leafed species results in a drastic increase in leaf complexity, whereas reduction of *KNOX1* expression in leaves can convert compound leaves to simple leaves [11–13].

PTS/TKD1 homologs were identified in several dicot species but not in rice and maize. Phylogenetic analysis showed that PTS/TKD1 orthologs formed an independent clade (PTS/TKD1) distinct from other KNOX proteins (Figure S4). The KNOX family proteins have three conserved domains [9, 14–16] (Figure 2H). The homeodomain is involved in DNA-binding activity [9, 14, 15, 17], and the ELK domain may repress the expression of a target gene(s) [14]. The MEINOX domain mediates protein-protein interactions [9, 17–19]. Interestingly, the predicted PTS/TKD1 protein contained a MEINOX domain, but an ELK domain and homeodomain were absent (Figure 2H). Thus, PTS/TKD1 homologs represent a novel dicot-specific subfamily of KNOX proteins that lack the homeodomain.

The Tomato Classical Mutant *bipinnata* Results from the Loss of Function of a *BEL-like homeodomain* Gene, *BIP*

The MEINOX domain mediates KNOX homodimerization and interactions with BELL transcription factors [9, 17–22]. The *A. thaliana* BELL proteins SAWTOOTH1 (SAW1) and SAWTOOTH2 (SAW2) interact with KNOX1 proteins and act redundantly to repress *KNOX1* expression in leaves [23]. *SAW1* and *SAW2* are expressed in lateral organs, including developing leaves, and the *saw1 saw2* double mutant has increased leaf serrations, resembling *KNOX1* overexpression phenotypes [23]. Interestingly, overexpression of the *A. thaliana* ortholog of *PTS/TKD1* resulted in a highly serrated phenotype resembling *saw1 saw2* double mutants (Figure S5). This phenotypic resemblance suggested the possibility that *PTS/TKD1* and *SAW* act in the same developmental pathway.

We hypothesized that mutations of tomato *SAW* would result in plants with extra leaf complexity, thus resembling the *Pts* phenotype. We searched for homologs of *SAW1* and *SAW2* in the tomato genome sequences and found a single bacterial artificial chromosome (BAC) (LE_HBa0111M10) containing the entire ORF of a tomato homolog of *SAW*. This BAC clone was mapped to the long arm of chromosome 2 (http://www.sgn.cornell.edu/). A classic tomato mutation *bipinnata*

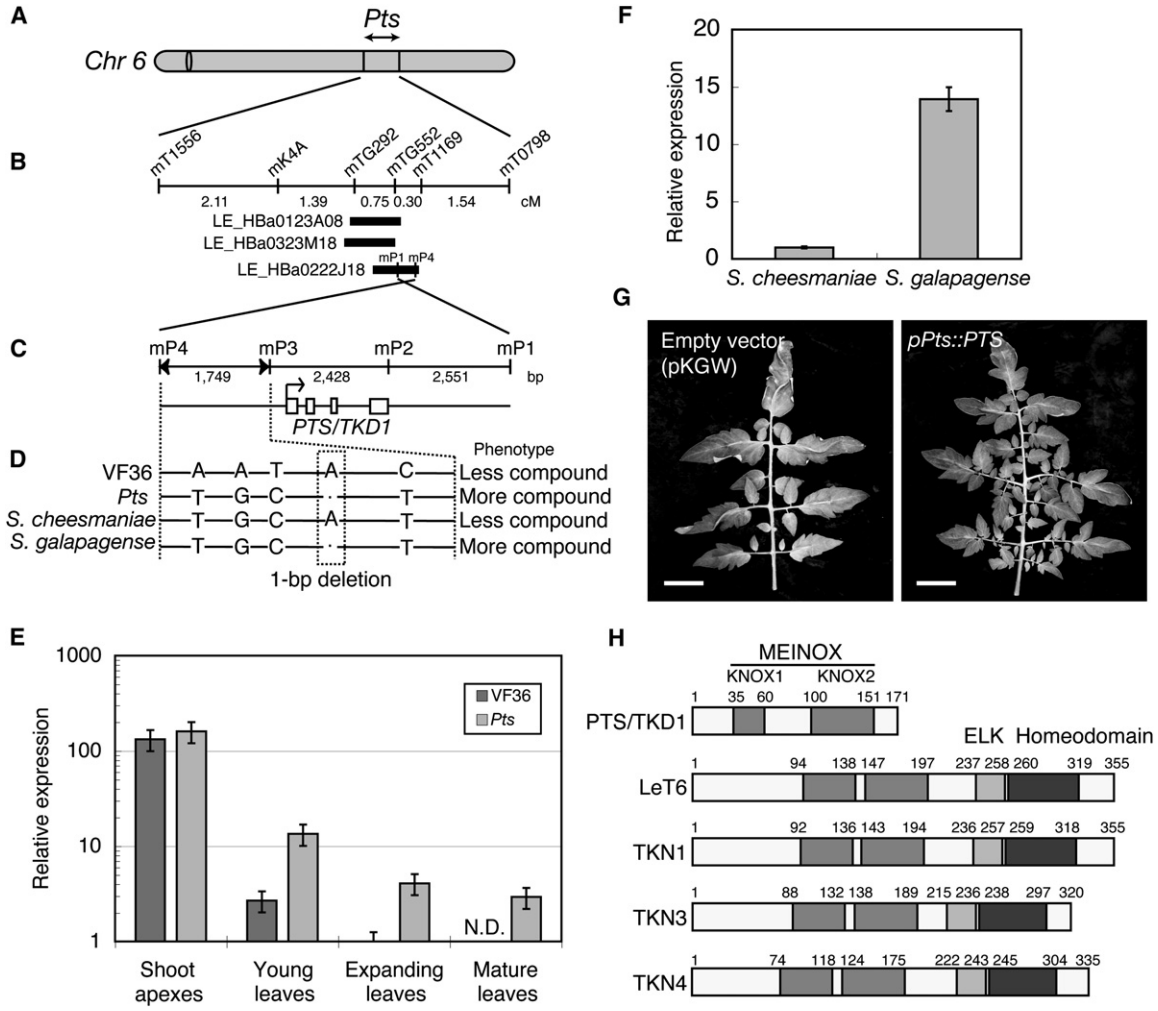

Figure 2. Map-Based Cloning of *Pts*

(A) Location of *Pts* on chromosome 6.

(B) Rough mapping of *Pts* locus. The relative positions of BAC clones are shown below the map.

(C) High-resolution linkage map of *Pts*. The number below the map indicates the physical distance (kbp) between molecular markers. The *Pts* gene structure is shown below the map.

(D) Comparison of genomic sequences of VF36, *Pts*, *S. cheesmaniae*, and *S. galapagense*.

(E) Expression of *PTS/TKD1* in the wild-type and *Pts*. Expression level was analyzed by QRT-PCR. Total RNA were isolated from shoot apexes, young leaves (5 to 10 mm), expanding leaves (25 to 35 mm), and mature leaves of 28-day-old plants. The expression level in expanding leaves of VF36 was set to 1. N.D. indicates not detected.

(F) Expression of *PTS/TKD1* in *S. cheesmaniae* and *S. galapagense*. Expression level was analyzed by QRT-PCR with total RNA isolated from expanding leaves. The expression level in *S. cheesmaniae* was set to 1. Error bars represent standard error (n = 3).

(G) Phenotype of transgenic tomato transformed with an empty vector (left) and with the *pPts::PTS* construct (right). Error bars represent standard error (n = 3). Scale bars represent 4 cm.

(H) Schematic illustration of PTS/TKD1 and other tomato KNOX1 proteins. The MEINOX (KNOX1 and KNOX2), ELK and homeodomain are represented by dark gray, light gray, and black boxes, respectively.

(*bip*) [6] (Figure 3A) closely resembles *Pts* phenotype and roughly maps to this position, suggesting that *bip* might represent a loss-of-function allele of *SAW.* Sequence analysis of the *SAW* ORF in *bip* revealed an 8 bp deletion introducing a premature stop codon (Figure 3D and Figure S6). No additional alleles of *bip* have been published, but two allelic mutants showed a *bip*-like phenotype [24] (Figures 3B and 3C) and had mutations in the *SAW* ORF (Figure 3D and Figure S6). Thus, the highly compound leaf of the classical mutant *bip* results from mutation of the tomato *SAW* gene, and we will henceforth refer to this gene as *BIP*. Like *SAW1* and *SAW2*, *BIP* is highly expressed in developing leaves and weakly expressed in the shoot apex (Figure S7). In the *A. thaliana saw1 saw2* double mutants, the *KNOX1* gene *BREVIPEDICELLUS* (*BP*) is overexpressed in leaves, and this overexpression is thought to increase leaf serration [23]. We found that *TOMATO KNOTTED-1* (*TKN1*; *BP* ortholog) was similarly overexpressed in the *bip* mutant (Figure 3E). The expression level of *TKN1* was also elevated in *Pts* and *S. galapagense* relative to the wild-type and *S. cheesmaniae*, respectively (Figures 3F and 3G). However, this is unlikely due solely to the leaf-proliferation phenotype of the *Pts* mutant because another *KNOX1* gene, *Lycopersicon esculentum Class1 KNOTTED-LIKE HOMEODOMAIN PROTEIN* (*LeT6*; *STM* ortholog), is not overexpressed in

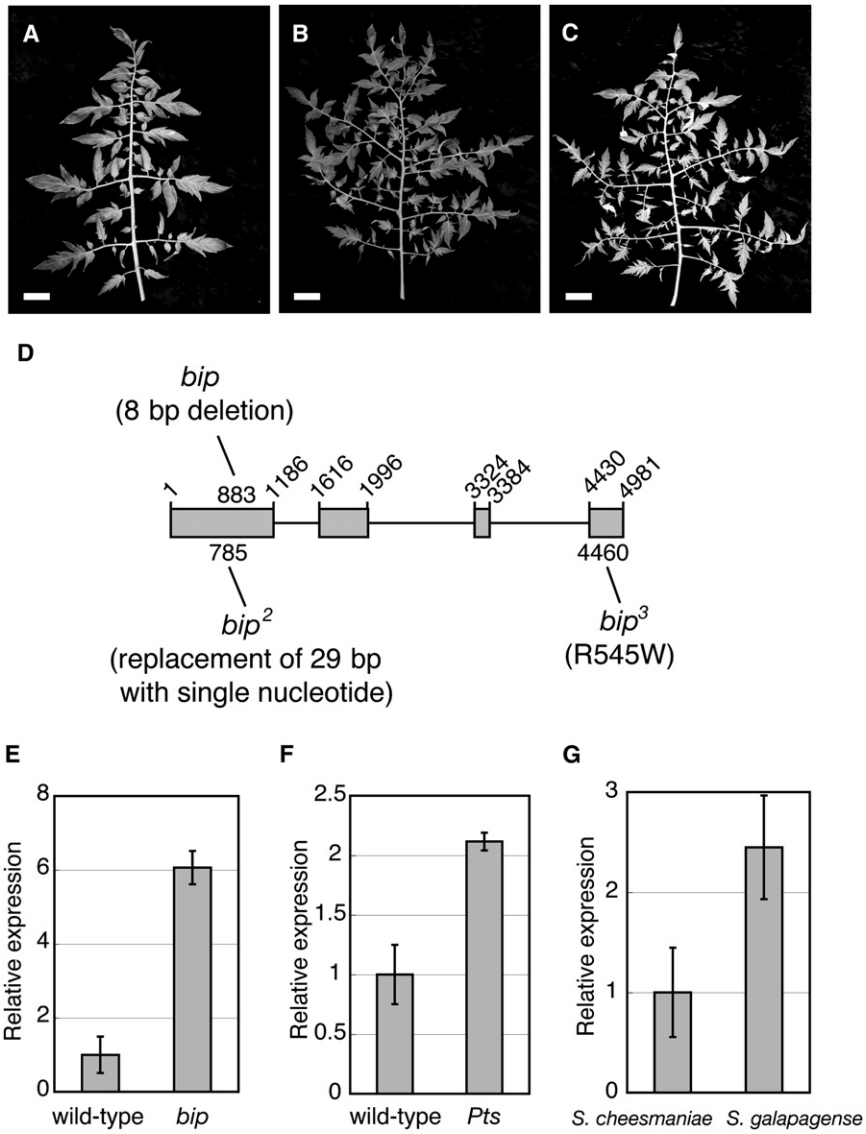

Figure 3. Cloning of *bip*

(A–C) Leaf phenotype of *bip* (A), *bip*^{2} (B), and *bip*^{3} (C). Scale bars represent 3 cm.

(D) Exon and intron structure of tomato *SAW*. The tomato *SAW* gene contains four exons (box) and three introns. The three mutants contain an 8 bp deletion (*bip*), a replacement of a 29 bp sequence with single nucleotide (*bip*^{2}), or an amino acid change in the highly conserved amino acid of homodomain (*bip*^{3}).

(E–G) Comparison of expressions level of *TKN1* between the wild-type (Ailsa Craig) and *bip* (E), the wild-type (VF36) and *Pts* (F), and *S. cheesmaniae* and *S. galapagense* (G). The expression levels in wild-type (in [E] and [F]) and *S. cheesmaniae* (in [G]) were set to 1. Error bars represent the standard error (n = 3).

Pts (Figure S8). These results suggest that overexpression of *PTS*/*TKD1* and loss of *BIP* function have the same downstream molecular (*TKN1* overexpression) and developmental (increased leaf complexity) consequences.

PTS Represses the KNOX1 Protein Interactions with BIP

Cooperative interactions of KNOX1-BELL complexes have been shown to mediate DNA-binding affinity and subcellular localization [17, 20, 22]. We hypothesized that PTS/TKD1 might compete with KNOX1 in binding to BIP. In two-hybrid assays, PTS/TKDI interacted with itself and BIP but not with the KNOX1 protein LeT6 (Figure 4A). We also tested the effect of the PTS/TKD1 protein on the interaction between LeT6 and BIP by two-hybrid competition assays (Figure 4B). LeT6 interacted with BIP, but this interaction was completely suppressed by induction of PTS/TKD1 expression (Figure 4B). Bimolecular fluorescence complementation (BiFC) was used to verify the interactions in planta and to determine the subcellular localization of the interacting complexes (Figures 4C–4J). Both PTS/TKD1 and LeT6 alone formed homodimers in the cytoplasm (Figures 4D and 4E), whereas BIP localized in both the nucleus and the cytoplasm (Figure S9). The LeT6-BIP complex was seen in the nucleus with some weak cytoplasm localization, suggesting that LeT6 presence in the nucleus is dependent on BIP (Figure 4F). Similarly, PTS/TKD1 interacted with BIP, but this complex was excluded from the nucleus (Figure 4G). LeT6-BIP complex formation was disrupted by PTS/TKD, confirming the results of the two-hybrid competition assay (Figures 4H–4J). These results indicate that PTS/TKD1 inhibits both BIP nuclear localization and LeT6-BIP complex formation in planta.

In *A. thaliana*, the SAW1 and SAW2 proteins repress *BP* expression and inhibit leaf lobing [23]. *BIP* loss of function results in *TKN1* overexpression and greater-leaf-complexity phenotype in tomato, suggesting a conserved role for the tomato *SAW* ortholog, *BIP*. One possible explanation for increased leaf complexity in *S. galapagense* (and *Pts*) is that overexpression of PTS/TKD1 causes disruption of the LeT6-BIP complex (with consequences similar to *BIP* and *SAW1* and *SAW2* loss of function). The other possibility is that disruption of the LeT6-BELL (including BIP) complexes makes LeT6 available to promote leaf complexity either on its own or through interaction with other as yet unknown proteins that are promoters of leaf complexity. In either case, it is likely that PTS acts to fine tune the relative proportions of these complexes. A complete understanding of the differing roles of KNOX proteins will likely require functional analysis of the unique activities of all KNOX-BELL complexes, as well as identification of other possible KNOX interaction partners.

Conclusions

Intense analysis of model organisms has revealed a central role for transcription factors in regulating developmental pattern. Alteration of expression of major developmental regulators often confers severe and pleiotropic phenotypes. From an evolutionary standpoint, the pleiotropic outcomes of modifying transcription-factor expression raises an

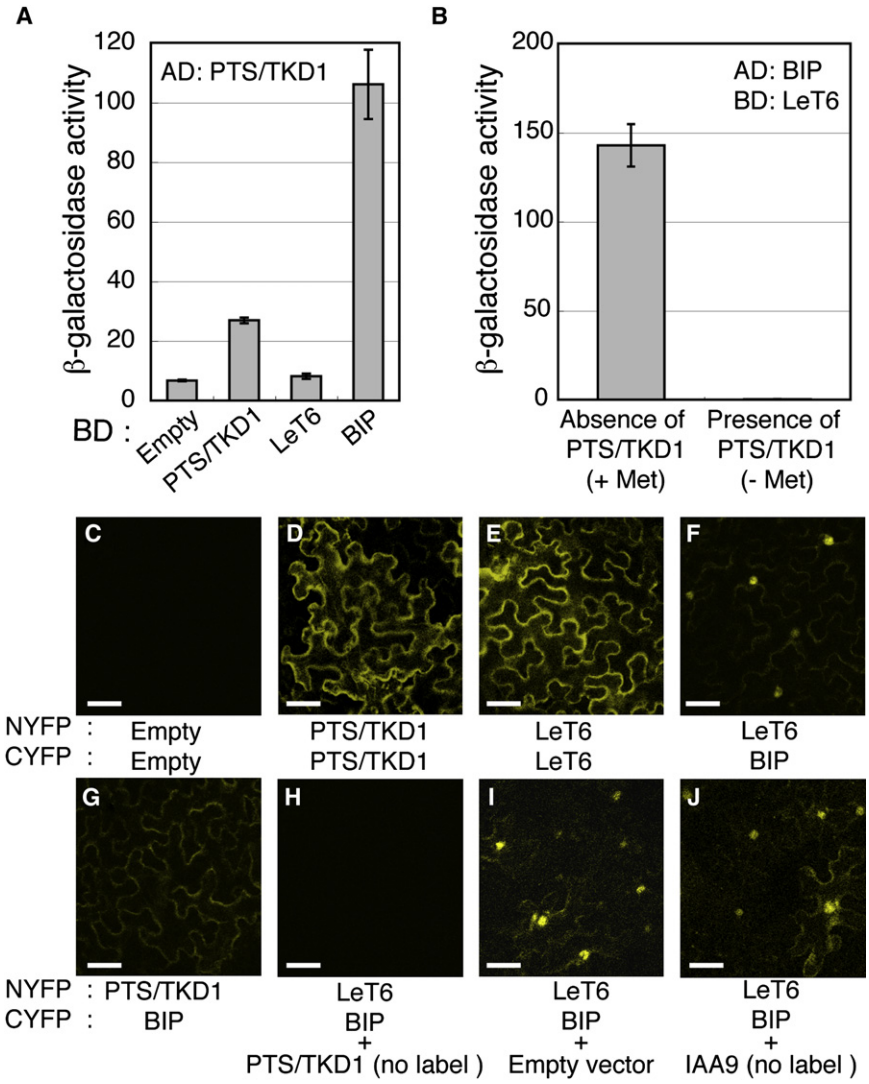

Figure 4. Analysis of Interaction between PTS/TKD1, LeT6, and BIP Proteins

(A) β-galactosidase assay for two-hybrid interactions. *LacZ* induction in transformed yeast was measured by the β-galactosidase assay with chlorophenol red-β-D-galactopyranoside (CPRG) as substrate. Error bars represent the standard errors (n = 5).

(B) Effect of PTS/TKD1 on the interaction between LeT6 and BIP. Two-hybrid competition assay was performed with pGADT7-BIP and pBridge-LeT6-PTS/TKD1. The interaction between LeT6 and BIP in the absence (+ Met) or presence (− Met) of PTS/TKD1 was measured by β-galactosidase assay.

(C–J) In planta bimolecular fluorescence complementation (BiFC). BiFC experiments were performed with pSPYNE-35S and pSPYCE-35S vectors. Combinations of vectors are shown below the pictures. Scale bars represent 40 μm.

interesting question: How can some phenotypic changes be programmed with these major developmental regulators without disrupting other developmental processes? In the case of *S. galapagense* and *S. cheesmaniae*, phenotypic variation may be accomplished by alteration of the dosage of one component of an intricate protein-protein interaction network governing KNOX1 activity in leaves. Mutations affecting the expression levels of transcription factors can modify the function of a major developmental regulatory complex in some organs without interfering with its other essential roles in morphogenesis. Such dosage-sensitive interactions may be broadly responsible for evolutionary change and provide a relatively simple mechanism for the generation of natural variation.

Accession Numbers

The GenBank accession numbers for *PTS/TKD1* and *BIP* are EU352653 and EU352654, respectively.

Supplemental Data

Experimental Procedures, nine figures, and one table are available at http://www.current-biology.com/cgi/content/full/18/9/672/DC1/.

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